RESEARCH ARTICLE

# An Ester Extract of Cochinchina Momordica Seeds Induces Differentiation of Melanoma B16 F1 Cells via MAPKs Signaling

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## Abstract

***Cochinchina momordica*** **seeds (CMS) have been widely used due to antitumor activity by Mongolian tribes of China. However, the details of the underlying mechanisms remain unknown. In the present study, we found that an EtOAc (ethyl ester) extract of CMS (CMSEE) induced differentiation and caused growth inhibition of melanoma B16 F1 cells. CMSEE at the concentration of 5-200 μg/ml exhibited strongest anti-proliferative effects on B16 F1 cells among other CMS fractions (water or petroleum ether). Moreover, CMSEE induced melanoma B16 F1 cell differentiation, characterized by dendrite-like outgrowth, increasing melanogenesis production, as well as enhancing tyrosinase activity. Western blot analysis showed that sustained phosphorylation of p38 MAP accompanied by decrease in ERK1/2 and JNK dephosphorylation were involved in CMSEE-induced B16 F1 cell differentiation. Notably, 6 compounds that were isolated and identified may be responsible for inducing differentiation of CMSEE. These results indicated that CMSEE contributes to the differentiation of B16 F1 cells through modulating MAPKs activity, which may throw some light on the development of potentially therapeutic strategies for melanoma treatment.**

**Keywords:** Cochinchina momordica seed - melanoma - B16 F1 - differentiation

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## Introduction

Current cancer therapies are often nonspecific with side effects, such as cell toxicities, hindered their clinical efficacy. A potentially less toxic approach to treat cancer is the agents that modify cancer cell differentiation, termed 'differentiation therapy', which has become a novel therapeutic approach aimed at modifying tumor cells proliferate at a slower rate and losing its earlier neoplastic attributes (Leszczyniecka et al., 2001; Serafino et al., 2004). Malignant melanoma represents one of the most treatment-resistant cancers of all human malignancies, with a median survival of 6-9 months (Prignano et al., 2002). It was reported that the incidence of melanoma is increasing more rapidly than that of any other cancer due to increased UV-ray intensities and artificial skin tannings (Park et al., 2010). Therefore, requirement for searching new drugs or novel combined chemotherapeutic protocols is urgent. Some agents have been reported to induce melanoma B16 cells differentiation, such as cAMP-elevating agents a-melanocyte-stimulating hormone (a-MSH), isobutylmenthylxanthine, forskolin, as well as the signal transduction pathway inhibitors LY294002, rapamycin and PD98059 (Prignano et al., 2006). However, the information of melanoma differentiation inducer concerning about the chemical compounds extracted from natural plants, is relatively limited up to date. Actually, in the search for new cancer differentiation inducer, the herbs being used in traditional medicines for cancer treatment are promising candidates.

*Cochinchina momordica* seed (CMS), the dried ripe seed of Momordica cochinchinensis (Lour.) Spreng. (Fam. Cucurbitaceae), was recorded in traditional Chinese medical book kai bao ben cao in Song dynasty. As a folk drug, it has a long history of application in a wide variety of diseases, including sores and inflammatory swelling, mastitis, scrofula, hemorrhoids, anal fistula, chronic eczema, neurodermatitis and scald. Recent studies showed that the extract of *Cochinchina momordica* seeds has potential effects on immune response and could be used as an adjuvant (Xiao et al., 2007). In addition, CMS has been widely used to treat various cancers in Inner Mongolian tribe areas of China even its underlying mechanisms have not been known yet. Our previous study (Zhao et al., 2010) has revealed that, the ethanol extract of CMS could potently inhibit the proliferation of lung carcinoma cells, breast carcinoma cells, esophageal carcinoma cells and melanoma cells in a dose-dependent manner. Among these tested cancer cells, mouse melanoma B16 F1 cell line was most sensitive to it. However, the anti-

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proliferation mechanism of CMSEE on B16 F1 cell, and what components in the CMS responsible for B16 proliferative inhibition are still unclear.

In the present study, we firstly demonstrated that EtOAc extract of cochinchina momordica seed (CMSEE) could suppress B16 F1 cells growth via inducing cells differentiation, and the chemical compounds from CMSEE were isolated and identified. Furthermore, we found that the activities of MAPK signaling pathway was involved in the CMSEE-induced differentiation.

## Materials and Methods

*Materials*

The mouse melanoma B16 F1 cells were obtained from the Cellular Biology Institute of the Shanghai Academy of Sciences (Shanghai, China), maintained in medium RPMI-1640 containing 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 $\mu$g/ml phytomycin, at 37 ºC in the humidified atmosphere of the 5% $CO_2$ incubator. For cells treatment, RPMI-1640 containing 1% FCS was used. RPMI-1640, fetal calf serum (FCS) and PBS were obtained from Gibco-BRL (life technologies, Paisley, Scotland). The specific ERK1/2-inhibitor (PD98059), p38 MAP kinase inhibitor (SB203580) and JNK kinase inhibitor (SP600125) were supplied by Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in DMSO (final maximal concentration of dimethylsulfoxide (DMSO) in medium was 0.1% [v/v]) to yield a 1mg/ml stock solution. Antibodies to total p44/42MAPK (ERK1/2), pho-p44/42 ERK1/2 (Thr202/Tyr204), pho-p38 MAPK (Thr180/ Tyr182) and pho-SAPK/JNK (Thr183/Tyr185) were all purchased from Cell Signaling Technology, Inc. (CST, CA, USA). Antibodies to total p38, total JNK, tyrosinase and GAPDH were supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

*Preparation of CMS extracts*

Plant material was purchased from Lerentang Pharmacy of Shijiazhuang (Hebei province, China) and authenticated by Professor Ren (New Drug Research and Development Center of North China Pharmaceutical Group Corporation, Shijiazhuang, China). After dried by baking and ground into fine powder, CMS samples(5kg) were soaked in 95% ethanol(15L) under reflux for 2×2 h. The extracts were combined and concentrated in vacuum at -40 °C. The concentrated extract was suspended in $H_2O$ and partitioned, individually with Water, petroleum ether (PE) and ethyl acetate (EtOAc) (1:20 W/V for all solvent). Of those, The EtOAc extract was namely CMSEE. The liquid phase was separated from the solid by filtration and concentrated by a rotary evaporator to dryness, and then redissolved in DMSO at 10 mg/ml for storage and using culture media without FCS for dilution to necessary concentration in the bioassays.

*Determination of cells viability, proliferation and apoptosis*

Cell viability was determined by the trypan blue exclusion method and proliferation rate was determined with the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium (MTT) assay. In brief, 10 µl of MTT (5 mg/ml) was added to each well in 96-well plates after treatments, and then incubation for 4 h at 100 µl of DMSO. The absorbance of each well was read at 570 nm using a kinetic micro plate reader. Cell viability was calculated by Trypan Blue. To investigate apoptosis, $1 \times 10^6$ cells were treated with CMSEE (40 µg/ml) for 0, 24, 48 and 72h. Cells were collected in PBS, in a final volume of 500 µl, incubated with annexin V-FITC and propidium iodide, as recommended (Apoptosis Detection kit, BD Biosciences) and analyzed by flow cytometry. In each experimental condition, $1 \times 10^5$ cells were analyzed. Data shown are mean ± S.E.M. of three independent experiments.

*Morphological and differentiation ratio analysis*

$5 \times 10^4$ cells were cultured in 35 mm dishes and treated with different dose of EtOAc extract for 48 h. After treatment, cells were fixed with 4% paraformaldehyde for 30 min at normal temperature, stained with Giemsa (0.1 % in glycerol and methanol) for 1min, and then with 0.025 % Giemsa staining solution for 15 min. For acridine orange-ethidium bromide (AO/EB) staining, cells were stained by AO (Acridine Orange, 50 µg/L) for 5 min and then stained with EB (Ethidium Bromide, 50 µg/L) for 10 min. All photographs were taken under light microscope and fluorescence microscope. Differentiation ratio (%) was expressed as the percentage of cells with cytoplasmic extension longer than three cellular bodies in relation to the total number of cells (Katya et al., 2008).

*Melanogenic contents and tyrosinase activity Assays*

Melanin release was measured as previously described (Mas et al., 2002). Briefly, cells were incubated with different concentrations of 20 µg/ml CMSEE or a-MSH for 24, 48 and 72 h, then washed with PBS and dissolved in 1.0 N NaOH for 1 h at 80 °C. The absorbance at 470 nm was measured, and melanin content was measured by using the authentic standard of synthetic melanin. Tyrosinase activity was assayed as described (Keishi et al., 2000; Yan et al., 2011) with minor modify. B16 melanoma cells were treated with 20 µg/ml CMSEE for 24, 48, 72 h, tyrosinase protein expression were determined by western blot. The cells were washed with ice-cold PBS and lysed by incubating at 4 °C for 30 min in lysis buffer (10 nM SDS, 150 mM NaCl, 1 mM EDTA, 0.5 Mm phenylmethylsulfony fluoride, and 10 µg/ml leupeptin). The lysates were centrifuged at 15, 000 × g for 30 min to obtain the supernatant as source of tyrosinase. The reaction mixture contained 50 mM phosphate buffer, pH 6.8, 0.05 % L-dopa, and the supernatant (tyrosinase). After incubation at 37 °C for 20 min, dopachrome formation was monitored by measuring absorbance at wavelength 492 nm.

*Western blot analysis*

Western blot was performed as previously described (Zhao et al., 2010). Briefly, lysates from B16 F1 cells were prepared with 500 µl of lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 Mm EGTA, Ph 8.0, 0.2 mM $Na_3VO_4$, 0.2 mM phenylmethylsulfonyl fluride, and 0.5% NP-40). The

protein was evaluated by using BCA assays and subjected to 10% SDS-PAGE, and electrotransferred to a PVDF membrane (Millipore). Membranes were blocked with 5% BSA for 2 h at room temperature, and incubated overnight at 4 °C with the primary antibodies as described individually in the figure legends. The bound primary antibody was detected by using appropriate fluorochrome-labeled secondary anti-rabbit or mouse IgG (IRDye 800-LI-COR, odyssey). After washed three times with TBS-T, membrane was imaged with Odyssey infrared imaging system (USA, LI-COR). The levels of protein were calculated as the ratio of the intensity of protein to that of GAPDH. Experiments were carried out in triplicate wells per time and repeated three times.

*Isolation and identification of chemical compounds from CMS*

CMSEE was subjected to column chromatography on PS25-300 with a successive elution system of 60% acetone, 70%acetone, and 80% acetone. The 70% acetone portion (4.6g) was subjected to Silica gel column and eluted with chloroform, chloroform-acetone 5:1, 3:1 and 1:1. The chloroform fraction was applied on a Pre-HPLC column (15% $CH_3CN$-$H_2O$, Phenomenex 250×21.2 mm, 10 μm) to obtain four pure compounds: MBZ-1, 2, 3 and 4. The chloroform-acetone 5:1 fraction was subjected to Silica gel column ($CHCl_3$-MeOH 30:1) and ODS C-18 column (40% MeOH-$H_2O$) to obtain MBZ-5, -6. The chloroform-acetone 3:1 fraction was subjected to Silica gel column ($CHCl_3$-MeOH 30:1) and Pre-HPLC column (19% $CH_3CN$-$H_2O$) to obtain MBZ-7, -8. The 80% acetone portion (3.2g) was subjected to Sephadex LH20 and eluted with a gradient of ethanol-water in proportion of 2:8, 4:6, 6:4, 8:2 and ethanol to afford MBZ-4.

All compounds were elucidated on the basis of ESI-MS (ZMD Micromass, Micromass, England) and NMR spectrometer (INVOA500, Varian, USA).

*Statistical analysis*

Data are reported as mean ± S.E.M. One-way analysis of variance (ANOVA) was performed to determine the significance between groups. Tukey's method was used for multiple comparisons. A P-value of less than 0.05 ($P < 0.05$) was considered as statistically significant. All the figures shown in this article were obtained from at least three independent experiments with a similar pattern.

## Results

*Inhibition of B16 F1 cell proliferation by various CMS extracts*

To investigate the effect of CMS extracts on the proliferation of B16 F1 cells, cells were individually treated with different CMS fractions (Water, PE and EtOAc) at the indicated concentrations (5, 10, 25, 50,100, 200 μg/ml) for 48 h. As shown in Figure 1, Water extracts showed inhibition of B16 F1 cells growth within 48 h of treatment in the dose of 100 and 200 μg/ml, and PE extract inhibited proliferation of B16 F1 cells at the concentration of 50-200 μg/ml. Nevertheless, the EtOAc extract, which was concentrated under vacuum to afford a viscous residue

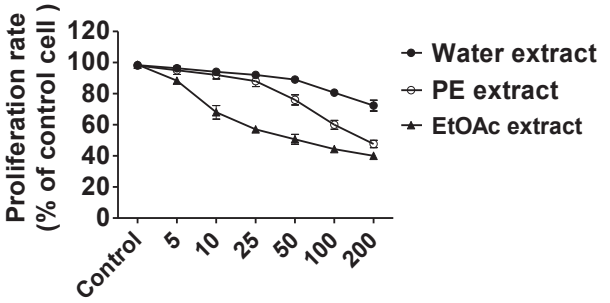


**Figure 1. Effect of Various CMS Extracts on B16 F1 Proliferation After 48 h Treatment.** Melanoma B16 F1 cells were treated with different extracts of CMS (Water, PE and EtOAc) or with medium for 48 h, after which cells were under MTT assay, the proliferation viability of medium-treated groups were recognized 100%. Values are the mean ± S.E.M (n=3)

16g, exert significant inhibition effect on B16 F1 cells in the all dose of 5-200 μg/ml, exhibiting the most potent growth arrest for B16 F1 cells. Therefore, we sought to elucidate the anti-cancer effects of the EtOAc extract (CMSEE) on B16 F1 melanoma cells in the following studies, and we will use the non-toxic concentration at 10-40 μg/ml.

*Growth arrest and differentiation of CMSEE induced melanoma B16 F1 cells*

To investigate the effect of CMSEE on the B16F1 cell proliferation, CMSEE were treated on B16F1cells with the indicated concentrations (5, 10, 20 and 40 μg/ml) for 24, 48 and 72 h. As shown in Figure 2A, B16F1cell proliferation was inhibited in a dose- and time-dependent manner. Moreover, Trypan Blue exclusion assay demonstrated that cell viability was more than 95% in all tested conditions (data not shown). Considering that dye exclusion assay could not detect initial stages of apoptotic death, which might underestimate a cytotoxic effect of CMSEE, binding 40 μg/ml CMSEE-treated cells to Annexin V was analyzed by flow cytometry after 24, 48 and 72h treatment. Results demonstrated that the cell apoptosis rate was not obvious different after CMSEE treatment (Figure 2B). In order to evaluate the mechanism of CMSEE on the anti-proliferation of melanoma cells, Giemsa and AO/EB staining were performed to investigate morphological changes. As shown in Figure 2C, 10-40 μg/ml CMSEE-treated cells obviously showed typical dendrite-like cellular protrusions, and the number of such elongated cells was significant, progressively increased according to the increase of CMSEE concentration. Accordingly, the dendrite form of cells was similar with that induced by 10 nM α-melanocyte-stimulating hormone (α-MSH), which could induce B16 cells differentiation as shown in reports (Keishi et al., 2000). Taken together, results above demonstrated that CMSEE-induced growth arrest of melanoma B16 F1 cells may result from inducing differentiation.

*Melanogenesis and tyrosinase analysis*

It is known that Melanogenesis is a principal parameter of differentiation in melanoma cells (Hearing et al., 1989). Thus, in the present study, melanin content was investigated to further verify CMSEE-induced cell

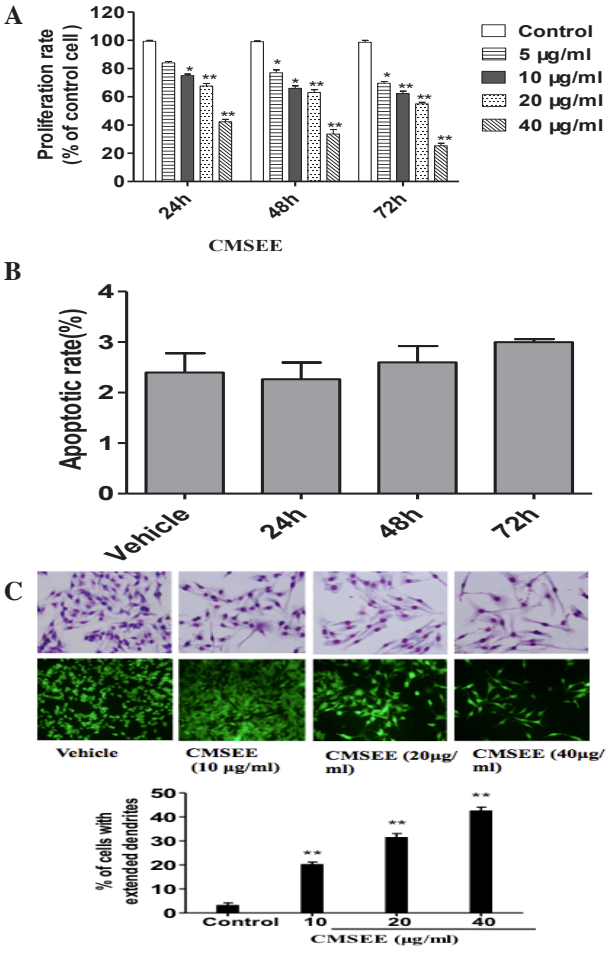


**Figure 2. CMSEE Induces Cell Growth Arrest, Apoptosis and Differentiation.** (A) Melanoma B16 F1 cells were treated with different concentrations of EtOAc extract or with medium for 24, 48 and 72 h, after which cells were under MTT assay, the proliferation viability of medium-treated groups were recognized 100%. Values are the mean ± S.E.M (n=3). **P < 0.01, *P < 0.05, compared with control. (B) Effect of CMSEE on the apoptosis of melanoma B16 F1 cells. Representative photographs of three independent experiments are shown. Early apoptotic cells were Annexin V-positive, PI-negative (lower on the right), and apoptotic and dead cells were defined as Annexin V-positive, PI-positive (upper). Data represent mean ± S.E.M in three separate experiments. (C) B16 F1 cells were treated with 10, 20 and 40 μg/ml CMSEE for 48h, then stained with Giemsa (upper part) or AO/EB (lower part). In each experiment at least 250 cells were counted (total number of cells), among which cells with dendrites longer than three cellular bodies were quantified. **P < 0.01, compared with control. Magnification 200×

differentiation. As shown in Figure 3A, 20μg/ml CMSEE increased melanin concentration significantly after 24 h, 48 h and 72 h treatment compared with vehicle group. Synthesis of melanin starts from the conversion of L-tyrosine to 3,4-dihydroxyphenylalanine (L-dopa), and then the oxidation of L-dopa yields dopaquinone by tyrosinase, the rate-limiting step enzyme in the melanin biosynthesis. Thus we examined whether CMSEE affected the activity of tyrosinase. As shown in Figure 3B, the tyrosinase activity increased in CMSEE-treated cells markedly. Furthermore, the expression level of the tyrosinase protein was enhanced in CMSEE-treated cells than that in control cells (Figure 3C), demonstrating that

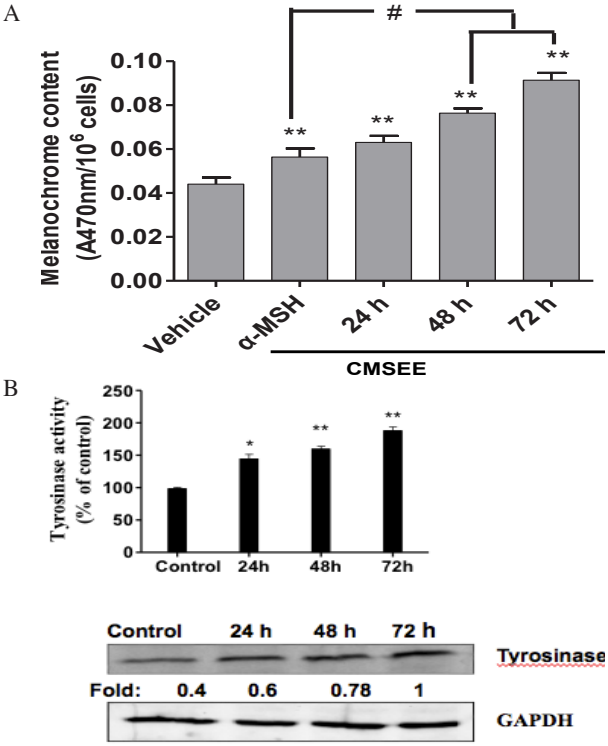


**Figure 3. Effects of CMSEE on Melanin Content, Tyrosinase Activity, and the Tyrosinase Protein Level in B16 F1 Melanoma Cells.** (A) The cells were incubated with 10 nM α-MSH and 10, 20 and 40 μg/ml CMSEE for 48h, melanin contents were measured in colorimetric way. Statistical analysis was performed by using Student's test. **P < 0.01, compared with control. #P <0.01, compared with α-MSH treatment group. (B) Tyrosinase activity was determined by measuring the formation of dopachrome as described as methods. Data represent the mean + S.E.M of three different experiments, each carried out in duplicate. **P < 0.01, * P < 0.05, compared with vehicle group (C) The level of tyrosinase protein in the melanoma cells was measured by Western blot analysis

CMSEE increased the melanogenesis partly by means of regulating tyrosinase protein level in B16 F1 melanoma cells. These results supported the idea that B16 F1 cells was in the process of differentiating to normal melanocyte cells.

*Effects of CMSEE on the activation of MAPKs pathway*

MAPKs signaling pathway is reported to be responsible for cell proliferation and differentiation of melanoma (Wong, et al., 1974; Landa, et al., 1998; Hirata, et al., 2007). To determine whether MAPKs pathway was involved in CMSEE-induced differentiation of B16 F1 melanoma cells. Western blot assay was performed to investigate the change of protein expression in MAPKs signal pathway. As shown in Figure 4, CMSEE significantly induced a time-dependent stimulation of p-p38 MAP whereas inhibited the activity of p-ERK1/2 and p-SAPK/JNK. Furthermore, changes in these kinases activities were slowly onset and peaked at 60 min for levels of p-p38 MAP and p-ERK1/2 proteins, but for that of p-SAPK/JNK at the time of 30 min, then gradually decreased. However, the total p38, SAPK/JNK and ERK level did not show significant changes after CMSEE treatment.

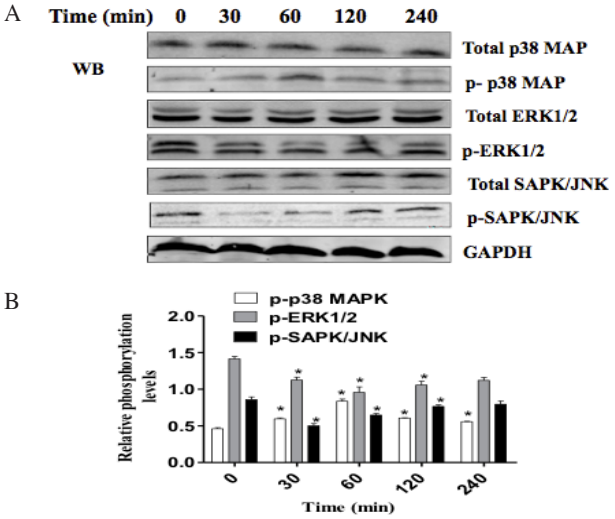


**Figure 4. Effects of CMSEE on the Proteins Expression in MAPKs Signal Pathway (ERK, JNK and p38).** B16 F1 cells were treated with CMSEE (40 μg/ml) for 30-240 min, or untreated. The protein levels were determined by means of Western blot analysis. (A) One representative gel of three independent experiments is as shown. (B) The band intensity of each group was determined via densitometry analysis, and was expressed as a relative density to GAPDH. The experiment was repeated at least three times with similar results. * $P < 0.05$, compared with vehicle group

*Effects of CMSEE combining with specific inhibitors of MAPKs families on B16 F1 cells proliferation, differentiation*

To further elucidate the underling mechanism of p38, ERK and JNK in CMSEE- induced cells differentiation, combination of specific MAPKs inhibitors and CMSEE on growth and differentiation of B16 F1 cells were investigated. Cells were pretreated with specific p38 MAP inhibitor (SB203580, 20 μM), MEK/ERK inhibitor (PD98059, 10 μM) or JNK-specific inhibitor (SP600125, 20 μM) for 15 min followed by treatment with or without 40 μg/ml CMSEE. The results were presented in Fig. 5(A), showing that PD98059 and SP600125 inhibited the proliferation of B16 F1 cells respectively, whereas SB203580 had no significant effects on it. Moreover, the combination of CMSEE with PD98059 or SP600125 enhanced the role of CMSEE-mediated growth arrest of melanoma B16 F1 cells while SB203580 blocked cells proliferative inhibition induced by CMSEE obviously. To further explain activities of MAPKs involved in CMSEE-induced melanoma cells differentiation, the synergistic effects of CMSEE with specific inhibitors on B16 F1 cells differentiation were investigated. As shown in Figure 5B-b and c, PD98059 and SP600125 induced differentiation of B16 F1 cells, respectively, whereas SB203580 alone did not affect B16 F1 differentiation. Moreover, the combination of CMSEE with PD98059 or SP600125 augmented the CMSEE-induced differentiation of melanoma B16 F1 cells. However, block of p38 MAP pathway by SB203580 markedly impeded the B16 F1 cells differentiation induced by CMSEE significantly (Figure 5B-a). Furthermore, PD98059 or SP600125 significantly augmented CMSEE-induced reduction of expression of p-JNK and p-ERK1/2 (Figure 5B-b and c). Taken together, the CMSEE-induced B16 F1 differentiation was

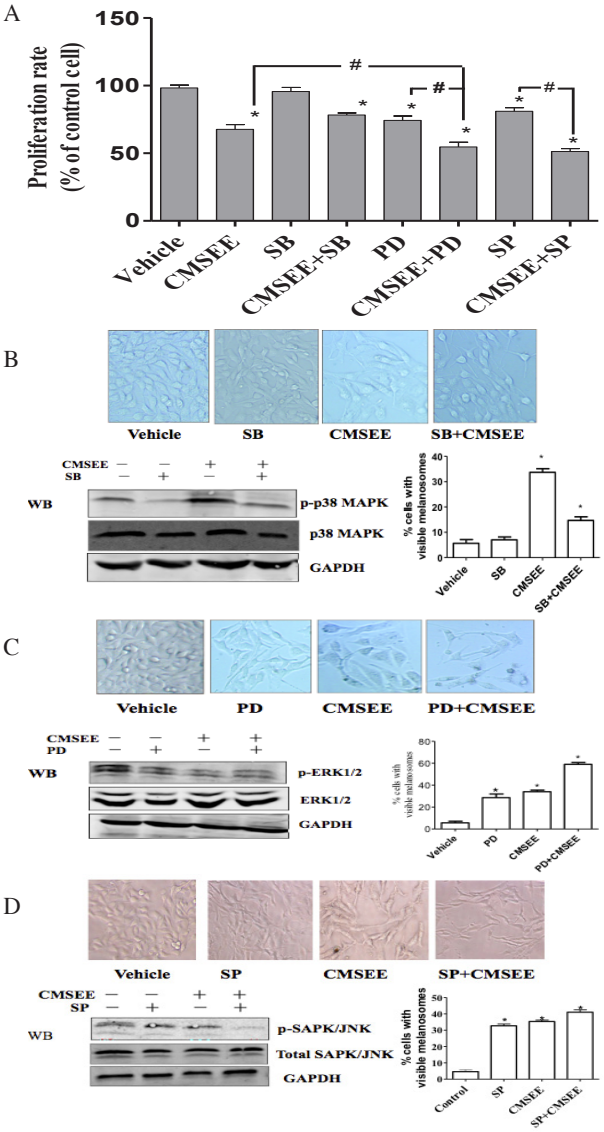


**Figure 5. Effects of Inhibitors of MAPKs on CMSEE-mediated Proliferation, Differentiation and Activity Changes of MAPK Kinase Family.** (A-a) Effects of inhibitors for JNK, ERK and p38 MAPK on B16 F1 cell proliferation. *$P<0.05$, compared with vehicle group. #$P < 0.05$ represents the relativity between two groups (n = 3). (B) When B16 F1 cells were cultured in medium up to 70 % confluence, a: SB203580 (20 μM); b: PD98059 (10 μM) and c: SP600125 (20 μM) were added to three culture flasks respectively, after co-cultured for 15 min, cells were then treated with CMSEE (20 μg/ml) for 48 h. Cell morphology was investigated under the light microscope, then total cell proteins were extracted and Western blot was performed

synergized, enhanced, and suppressed by the MAPKs activities.

*The chemical compounds identification*

To further explore which constituents or what chemical compounds of CMS are responsible for CMSEE-induced B16 F1 differentiation. We used HPLC-ESI-MS and NMR spectrometer analysis to isolate and identify the active compounds as described as methods. Finally, 8 compounds were got in pure Chloroform-eluted fraction. Their structures were identified based on spectral analysis (MS, 1H NMR, 13C NMR), namely as p-hydroxylcinnamaldehyde, coniferylaldehyde,

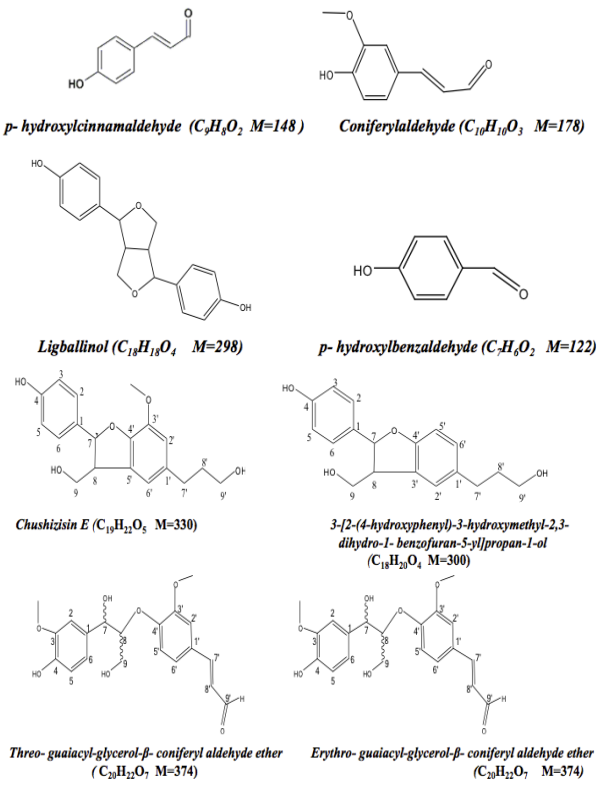


**Figure 6. Structures of Chemical Compounds Extracted from CMSEE**

ligballinol, Chushizisin E, 3-[2-(4-hydroxyphenyl)-3-hydroxymethyl-2,3-dihydro-1-benzofuran-5-yl] propan-1-ol, erythro-guaiacyl-glycerol-β-coniferyl aldehyde ether and hreo-guaiacyl-glycerol-β- coniferyl aldehyde ether. as shown as in Figure 6. Since the dendritic phenotype of B16F1 cells was the marker of differentiation. To address whether the above compounds were responsible for CMSEE-mediated B16F1 differentiation, we performed Giemsa staining after treated with 8 compounds respectively. We found that compounds p-hydroxylcinnamaldehyde, ligballinol, Chushizisin E, 3-[2-(4-hydroxyphenyl)-3-hydroxymethyl-2,3-dihydro-1-benzofuran-5-yl]propan-1-ol, erythro-guaiacyl-glycerol-β-coniferyl aldehyde ether and hreo-guaiacyl-glycerol-β- coniferyl aldehyde ether could induce B16F1 cells morphology that analogous to that observed treated by CMSEE (data was not shown), thus these 6 compounds all have the effect of inducing B16 F1 cells differentiation. Furthermore, we analyzed concentrations of them induce 50% cells differentiation. They were 4.23±0.32, 25.41±1.47, 46.00±4.23, 40.01±3.29, 80.61±3.27 and 82.09±5.15 respectively. Therefore, we found that p-hydroxylcinnamaldehyde may be the most powerful chemical compound that induces B16 F1 cells differentiation than others.

## Discussion

In the present study, we investigated the anti-proliferation effect of CMSEE on the melanoma B16 F1 cells. We found that the proliferation of B16F1 cells was significantly inhibited by EtOAc extract (CMSEE) more than PE and Water extract. Therefore, we sought to elucidate the anti-cancer effects of CMSEE on B16 F1 melanoma cells, and tried to identify the active ingredients in CMS responsible for the differentiation effects.

Recently, studies of cancer cell differentiation provided useful information for the preclinical evaluation of potential anti-tumor drug. However, the information concerning solid tumor cells differentiation inducers is relatively limited compared with those that induce leukemia cells differentiation (Davis, 2000; Leszczyniecka et al., 2001). Melanoma cell is a well available cell model for screening solid tumor cell differentiation inducers, since its differentiation is generally accepted to be accompanied by slower cell proliferation, dendritic cell morphology (Wilkinson et al., 2000; Puri et al., 2004; Panich, et al., 2012) and increased melanin production through long dendritic process (Wikinson, et al., 2000). In the search for new cancer therapeutics, the herbs being used in traditional medicines for cancer treatment are promising candidates, but there were a few reports with regard to cancer cells differentiation inducer compared with that induced apoptosis. Lupane triterpenes isolated from Chinese Dandelion Root has reported to induce B16 2F2 differentiation (Hata et al., 2006). In addition, the ethyl acetate of Phellinus linteus grown on panax ginseng could inhibit B16 F10 cell proliferation by means of inducing apoptosis and cellular differentiation (Jiang et al., 2009). These reports showed that Chinese herb is the promising resources of differentiation inducer. In our results, CMSEE inhibited proliferation of melanoma cells through inducing cell differentiation that is characterized by proliferation inhibition, dendrite-like outgrowth, increased melanogenesis production, and enhanced activity of tyrosinase as shown in results, which are consistent with some previous reports (Gruber, 1992). Tyrosinase (TYR), a type I membrane protein and copper-containing enzyme, is involved in the production of melanin, the primary pigment found in vertebrates. Tyrosinase involves two key steps in the melanin synthesis pathway through catalyzing the hydroxylation of L-tyrosine to L-DOPA followed by the oxidation of L-DOPA to dopaquinone (Halaban, 2001). We observed that CMSEE significantly up regulated the Tyrosinase activity and protein level in B16 F1 cells, which might be due to the outcome of cellular differentiation.

To further investigate the differentiation-induced mechanism of CMSEE in B16F1 cells, we analyzed the changes of protein in MAPKs signaling pathway. It is known that Mitogen-activated protein kinases, including extracellular signal regulated kinase (ERK), c-jun amino-terminal kinases (JNK), p38 MAPK kinase or Erk5/BMK, have been identified to play specific, albeit cross-talking roles in the regulation of fundamental cellular function and important roles in diverse cellular processes, such as cell proliferation death, migration, and differentiation (Aouadi etal., 2006; Krishna and Narang, 2008; Eralp et al., 2008; Mantha and Jumarie, 2010). Moreover, accumulation studies demonstrated that MAPKs was involved in the differentiation of various cancer cells (Wang etal., 2006; Chang et al., 2007; Sundaramurthy et al., 2009), including melanoma cells differentiation (Selimovic et al., 2008).

ERKs (ERK1 and ERK2) are activated by MEK and are generally thought to regulate cell growth and

differentiation whereas JNK and p38 are considered to regulate apoptosis and response to stress in the past years. However, recent studies have suggested that this was not always the case. Activation of ERK has been shown to protect certain cells from the induction of apoptosis by various agents whereas activation of JNK or p38 kinases has been reported to stimulate apoptosis or differentiation (Englaro et al., 1995; Daniel et al., 2004). Therefore, cells survival and differentiation appear to require the proper balance of activation and inhibition of the MAPK signaling molecules. Some differentiation induction agents were suggested to associate with MAPKs activities. Nagata (Nagata et al., 1998) indicated that activation of p38 and JNKs cascades but not of ERKs were required for Epo-induced erythroid SKT6 cells differentiation. Ding (Ding et al., 2001) showed that NaBT-induced differentiation and apoptosis in intestinal cells Caco-2 and HT29 were associated with inhibition of ERK and JNK pathway. Here we suggested that CMSEE-induced differentiation depended on inhibition of ERK or JNK kinase activity and stimulation of p38 activity. As indicated role of MAPKs activity in differentiation, the MAPKs specific inhibitors PD98059, SB302580 and SP600125 were used to evaluate this conclusion. ERK/MEK inhibitor PD98059 and JNK inhibitor SP600125 decrease significantly the ability of MEK1/2 to phosphorylate ERK1/2 and the ability of SAPK to phosphorylate JNK. In this study, CMSEE also suppressed the phosphorylation of ERK and JNK, leading to B16 F1 differentiation. In addition, p38 MAPK inhibitor SB203580 had no effect on B16 F1 differentiation while it impeded CMSEE-induced differentiation. These results suggested that CMSEE modulated proper balance of activation of the MAPK signaling pathway and induced B16 F1 cells differentiation finally. Further study is required to analyze the activity changes of proteins such as Ras, PKC, PKA and cAMP in the upstream of MAPKs and the proteins in the downstream, to determine the target of CMSEE in the B16F1 cells.

In the present study, we found that CMSEE inhibited B16F1 melanoma cell proliferation stronger than Water and PE extract. Therefore, we chose EtOAc extract as our test sample. Furthermore, we isolated and clarified 8 compounds that are included in a large quantity in the CMSEE, MBZ-1, 2, 3, 4, 5, 6, 7, 8 namely p-hydroxylcinnamaldehyde, Coniferylaldehyde, p-hydroxylbenzaldehyde, ligballinol, ChushizisinE, 3-[2-(4-hydroxyphenyl)-3–hydroxymethyl-2,3-dihydro -1-benzofuran-5-yl]propan-1-ol, erythro-guaiacyl-glycerol-β-coniferyl aldehyde ether and hreo-guaiacyl-glycerol-β- coniferyl aldehyde ethe. Of these compounds, we sought that 6 of them can induce differentiation on melanoma B16 cells, they are p-hydroxylcinnamaldehyde, ligballinol, ChushizisinE, 3-[2-(4-hydroxyphenyl)-3–hydroxymethyl-2,3-dihydro -1-benzofuran-5-yl]propan-1-ol, erythro-guaiacyl-glycerol-β-coniferyl aldehyde ether and hreo-guaiacyl-glycerol-β- coniferyl aldehyde ethe. However, the exact effect and mechanism of these chemical compounds on proliferation and differentiation of B16F1 cells are required to be elucidated. We will investigate whether these chemical compounds are responsible for the activity of CMSEE. In addition, we should explore whether there are other chemical compounds included in CMSEE exert differentiation effect on B16F1 cells in the subsequent study.

In summary, it was firstly reported in the present study that CMSEE, as an anti-tumor drugs, induce differentiation of mouse melanoma B16 F1 cells via MAPK signal pathway. Furthermore, six chemical compounds potential responsible for CMSEE-induced differentiation were isolated and clarified. However, some other important issues remain unresolved about the differentiate effect of CMSEE, particular about how these compounds in CMSEE interact to trigger cancer cells the differentiate signals, and whether CMSEE and the compounds in it decrease the volume and weight of bearing tumor in vitro. Resolving these issues will help establish the physiological mechanisms for antitumor functions of a valuable and traditional medicinal CMS. Further studies concerning about these issues are on going now.

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